Evaluation of CMV-specific cellular immune response by EliSPOT assay in kidney transplant patients.

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Short title. CMV-immune response in kidney transplantation.

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ABSTRACT

Background. Immunological monitoring for CMV can be useful in transplant patients; however, few centers perform it on a routine basis.

Objectives. In this study, CMV-specific cellular response was evaluated in a population of kidney transplant recipients and related to viral infection/reactivation and other demographic and clinical features.

Study design. Three-hundred-twenty-eight patients were studied by EliSPOT assay: 201 prospectively monitored in the first year posttransplantation, 127 with a single determination at >1 year. Clinical features, including occurrence of CMV-DNAemia, CMV serostatus, anti-viral strategies and immunosuppressive protocols, were evaluated.

Results. Overall, 66.5% of patients were CMV- responders at EliSPOT assay. No episode of infection occurred at follow-up (mean 24.5 months) in 73.4% responders versus 55.5% non-responders ($p < 0.005$); CMV-free period was significantly longer in responders ($p<0.001$). Although no significant difference of peak viral load was found, prevalence of CMV-DNAemia values $>10^5$ copies/mL was significantly higher in non-responders versus responders (8.2% and 2.3%, $p<0.05$). Non-responder status was significantly associated to CMV-seronegativity ($p < 0.0001$), anti-viral prophylaxis use ($p < 0.0001$), and immunosuppression induction with basiliximab ($p < 0.005$). No significant association was found for other clinical features and immunosuppressive protocols.

Conclusions. Immunological data for CMV could be used in the clinical evaluation and decision-making process, in combination with virological monitoring, in kidney transplant recipients.

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Keywords: cytomegalovirus; cellular immune response; EliSPOT assay; CMV-DNAemia; kidney transplantation.

Background

In transplant patients, Cytomegalovirus (CMV) may reactivate from latency due to immunosuppression or cause primary infection in seronegative recipients. In kidney transplantation (KT), CMV infection and disease have been reported in 8%-32% and 8%, respectively [1]; moreover, CMV has been associated to indirect effects, including rejection, chronic nephropathy, and other opportunistic infections [1-3]. As CMV-specific T-cell response has been associated to decreased rates of infection/disease [4-11], its evaluation may be valuable in combination with viral monitoring, according to the Updated International Consensus Guidelines on the Management of Cytomegalovirus in Solid Organ Transplantation [12]. Assays for immunological evaluation include intracellular cytokine staining, MHC multimer staining, QuantiFERON-CMV, and EliSPOT. An ideal assay should evaluate CD4+ and CD8+ T-cell response, optimally by measuring interferon (IFN)-γ, and should be simple, rapid, cost-effective, and reproducible. At moment, no assay is standardized with the exception of the QuantiFERON-CMV that, however, only evaluates CD8+ responses. Other limitations include the need for a flow cytometer (intracellular cytokine staining, MHC multimer staining) and HLA restriction (MHC multimer staining). EliSPOT enumerates IFN-γ-secreting mononuclear cells (both CD4+ and CD8+, without differentiating) in response to stimulus with CMV peptides and seems to represent a reproducible tool for monitoring T-cell activity *ex-vivo* [12]. Current evidence suggests that viro-immunogical evaluation can predict the risk of CMV viremia and disease in the postprophylaxis and preemptive context [4-11]. At moment, very few Italian centres perform CMV-specific immunological evaluation and its implications in the clinical decision-making process are poorly defined. The Turin Renal Transplant Centre is the first in Italy for activity volume (>100 KT/year).

Objectives

To evaluate CMV-specific cellular immune status in KT patients on a routine basis and investigate the association to viremia, demographic and clinical features.

Study design

Three-hundred-twenty-eight consecutive KT recipients (M/F, 218/110; mean age, 54.7±14.2 years; range, 28-75) were investigated in a mixed prospective-cross sectional study: 201 prospectively monitored in the first year posttransplantation and 127 at >1 year (up to eight). Main features of study population are summarized in Table 1. Informed written consent was obtained from all patients; the study was conducted in accordance with the ethical standards and Helsinki Declaration and approved by the Institutional Review Board. According to our centre's practice, virological monitoring was performed by quantification of CMV-DNAemia on whole blood (using a commercially available real-time PCR assay [CMV-ELITe MGB® kit, ELITechGroup, Milan, Italy]) twice weekly in the first month, twice monthly up to 3 months, every three months up to 1 year, and yearly thereafter. Further specimens were collected in the presence of CMV-DNAemia, usually within 7 days. Immunological evaluation was scheduled at 30, 60, 90, 180, and 360 days in the first year posttransplantation; and once at any time point at >1 year. No baseline immunological evaluation was made and no further specimens were collected in the presence of CMV-DNAemia. However, due to missing sending or specimen unsuitability (i.e. insufficient number of cells, invalid positive or negative control; see below for details), only 705 samples were available from the 201 patients evaluated in the first year posttransplantation (mean, 3.5/patient), in addition to 127 specimens from as many patients at >1 year, accounting for an overall number of 832 specimens. Data of CMV-DNAemia were available for all patients (median time of follow-up 24.5 months, range 24-42). Anti-CMV prophylaxis was administered for 3 months in high risk patients (i.e. donor/recipient seromatching, D+/R-, N=30)[13]. Pre-emptive treatment with ganciclovir or valganciclovir was administered in case of CMV-DNAemia $>10^4$ copies/mL or based on clinical judgment.

EliSPOT was performed as described elsewhere [14]. Briefly, automated separation of T cells from fresh blood samples was performed with the RoboSepR instrument (StemCell Technologies, Vancouver, Canada) using the EasySep™ Whole Blood T Cell Enrichment kit for immunomagnetic negative selection (StemCell Technologies), following the manufacturer's instruction. This system isolates cells from HetaSepTM-treated (ratio 1:5; StemCell Technologies) whole blood by targeting unwanted cells for removal with Tetrameric Antibody Complexes recognizing CD14, CD16, CD19, CD20, CD33, CD36, CD41, CD56, CD66b, CD123, glicophorin A and dextran-coated magnetic particles; the labeled cells are separated using the EasySep™ magnet, whereas desired cells are poured off into a new tube. According to manufacturer's, this system allows for an enrichment in CD3+ fraction (approximately from 11% to >96%), with recovery of also dendritic cells and a minimal amount of other cells, such as macrophages and B lymphocytes, functioning as antigen presenting cells. No further method to assess specimen purity was used. Separated cells were resuspended in RPMI-1640 medium (supplemented with 1% L-glutammine and 10% fetal calf serum). An aliquot of $2x10^5$ CD3+ cells (100 μL/well from a $2x10^6$/mL mix) was incubated for 20-24 h on an anti-IFN-γ antibody-coated plate (EliSPOT Interferon-γ Basis Kit; Autoimmun Diagnostika, Strassberg, Germany) with CMV-specific peptide mix (CMV-Spot ELSP5530, including pp65 and IE-1; Autoimmun Diagnostika), medium alone (negative control) or phytohemagglutinin (positive control). IFN-γ production was visualized by an enzyme-labeled detection antibody, with each spot representing a single cell secreting IFN-γ. Results were analyzed using a computer-assisted system (AID EliSPOT Reader System, Autoimmun Diagnostika). Background was subtracted for all the results (sample minus negative control). Results were expressed as spot forming units (SFU)/$2x10^5$ CD3+ cells. Specific immune response was evaluated as previously described [15]: invalid assay >5 SFU for the negative control and <20 for the positive control; absent/weak response <20, strong response ≥20 (accordingly, non-responders and responders patients).

Demographic and clinical variables were evaluated by univariate analysis and subsequently included in a multivariate logistic regression analysis. The t test was used for comparisons of quantitative variables between groups. Time to development of CMV reactivation was evaluated by Kaplan-Meier curve analysis. Evaluation of an intersection point with high specificity and sensitivity to differentiate patients with and without occurrence of CMV viremia in the subsequent 3 months on the basis of SFU values was made by ROC curve analysis. A commercially available software was used (IBM SPSS Statistics version 21). A p value <0.05 was considered statistically significant.

Results

Overall, 218/328 (66.5%) patients were CMV-responders (median SFU/2x10^5 CD3+ cells 131; range, 20-500); in particular, 125/201 (62.2%) individuals evaluated in the first year posttransplantation (in this subgroup of patients, immunological status was defined considering the whole period of study for each individual), with restoration of immune response within 6 months, and 86/127 (67.7%) at >1 year. In Figure 1, rate of responders and median SFU/2x10^5 CD3+ cells at different time points are reported. At least one episode of viremia occurred in 107/328 (32.6%) patients at follow-up: 58/218 (26.6%) responders versus 49/110 (44.5%) non-responders (p=0.002); with repeated episodes of infection occurring in 13/218 (6.0%) versus 18/110 (16.4%; p=0.005), respectively (Figure 2a). Viral load (peak level) tended to be higher in non-responders, although difference was not significant (mean±standard deviation: 1.1x10^5±3.4x10^5 and 3.8x10^5±8.4x10^5 copies/mL in responders and non-responders, respectively; p >0.3)(Figure 2b). However, a significantly higher prevalence of DNAemia values >10^5 copies/mL was found in non-responders versus responders (9/110, 8.2% versus 5/218, 2.3%, respectively, p<0.05)(Figure 2c). Kaplan-Meier analysis evidenced that CMV-free cumulative incidence was significantly higher in responders versus non-responders (p<0.001; Figure 3). No case of CMV disease occurred at follow-up.

Subsequently, relation between demographic and clinical features and CMV-specific immune status and viremia was evaluated (Table 2). At univariate analysis, non-responder status was significantly associated to male gender ($p<0.05$), seronegative recipient status (R-) at transplantation ($p<0.0001$), antiviral prophylaxis ($p<0.0001$), and immunosuppression induction with basiliximab ($p<0.005$); whereas no association was found for other features, including age, time from transplantation, immunosuppressive induction with anti-thymocyte globuline and immunosuppressive protocols including calcineurin-inhibitors and mTOR-inhibitors.

In particular, 28/39 (71.8%) R- patients were non-responders irrespective of donor serostatus, whereas the remaining R- individuals developed a CMV-specific cellular immune response in the first year posttransplantation following primary infection. Among D-/R- patients, one subject developed a primary infection at 1 month (viral load, 2,071,000 CMV-DNA copies/mL). As expected, EliSPOT evidenced a non-responder status in the first months posttransplantation, with development of CMV-specific response at 6 months (20 SFU//2x10^5 CD3+ cells). For the analysis, this patient was included among the responders, however this level of response was only partially maintained (subsequent EliSPOT values between 12 and 20). On the other hand, 207/289 (71.6%) R+ patients displayed a responder status ($p<0.001$).

High risk patients (D+/R-) on antiviral prophylaxis usually exhibited a non-responder status, as expected; while pre-emptive treatment was associated with recovery and presence of CMV-specific cellular immune response ($p<0.001$).

As regards immunosuppression, only induction with basiliximab was significantly associated to a non-responder status. It is to note that anti-thymocyte globulin was used only in six patients, five of which reconstituted CMV-specific cellular immune response within three months.

Multivariate analysis of demographic and clinical features in relation to CMV non-responder status, evidenced a significant association for R- serostatus ($p<0.0001$; hazard ratio [HR] 13.02, 95% confidence interval [CI] 5.21-32.5) and male gender ($p<0.005$; HR 2.40, 95% CI 1.36-4.24).

Among demographic and clinical features evaluated at univariate analysis for the occurrence of CMV viremia (Table 2), a significance was found only for age >50 years and induction with basiliximab. No factor resulted significantly associated at multivariate analysis.

Figure 4 illustrates ROC curve analysis for SFU/2x10^5 CD3+ cells values in terms of occurrence of CMV-DNAemia in the subsequent 3 months.

Discussion

In the present study, CMV immunogical data were routinely investigated in KT recipients by EliSPOT, in contrast to previous studies performed on selected or voluntarily recruited patients and more often by QuantiFERON-CMV assay. The optimization of the method with automated separation of total CD3+ cells could improve specificity of this application, as EliSPOT usually enumerates IFN-γ secreting mononuclear cells without distinguishing between NK and T cells.

Although the lack of baseline immunological data should be considered, most of the patients evidenced recovery of CMV-specific T-cell response within the first months posttransplantation: 53.1% (median, >70 SFU/2x10^5 CD3+ cells) at 3 months up to approximately 65% (median, >100) at 6 months. Response was persistent throughout the follow-up with no subsequent relevant increase in the total rate of responders and level of response. At follow-up, recovery of response was significantly associated to a lower incidence of viremia (26.6% versus 44.5% in responders and non-responders, respectively). In responders, episodes of infection were characterized by low level CMV-DNAemia (< or slightly >2x10^3 copies/mL, limit of detection of the real-time PCR assay) and short duration (negative on subsequent evaluation, in the absence of antiviral administration). In non-responders, occurrence of CMV-DNAemia was significantly higher, in particular repeated episodes of infection; the higher occurrence of values >10^5 copies/mL suggests a potential impact on uncontrolled replication. It is to note that antiviral administration was based on clinical judgment and/or CMV-DNAemia values >10^4 copies/mL. It has been hypothesized that a certain level of viral

196 replication is required to stimulate an adequate immune response: CMV-DNAemia from 7000
197 copies/mL in R- patients when prophylaxis was discontinued in a previous study [7]. On the other
198 hand, the prompt administration of antivirals could interfere with immunological boost by reducing
199 viral exposure. This could explain the significantly higher frequency of repeated episodes of
200 reactivation in non-responders, as no sufficient exposure was accomplished by administrating the
201 antiviral agent for low viral loads.
202 Considering serological matching and antiviral strategy, prophylaxis-treated D+/R- patients did not
203 usually mount an adequate response (73.3% non-responders up to 1 year posttransplantation), as
204 previously reported [7]. Despite of the high effectiveness of prophylaxis, primary infection may
205 occur, thus probably determining the priming of CMV-specific cellular response. This is well
206 recognized in KT (38% of 13 seronegative patients in the study by Abate and coll. [7]), as well as in
207 lung transplantation [16,17]. Priming of response can also occur in D-/R- patients following a
208 community-acquired infection. Seronegative status at transplantation and prophylaxis were among
209 the few factors significantly associated to the lack of an adequate response at univariate analysis;
210 association to seronegativity was highly significant also at multivariate analysis.
211 Considering immunosuppression, induction with basiliximab was significantly associated to non-
212 responder status and viremia in our study, in contrast with previously reported data. Basiliximab is
213 an IL-2 receptor antagonist that intervenes in a critical pathway involved in allograft rejection, thus
214 impairing the immune response to antigenic challenges. Nevertheless, previous studies have
215 reported similar incidence of CMV infection in basiliximab-treated patients and controls (17.3%
216 versus 14.5%) [18]. It could be hypothesized that other factors such as serostatus, antiviral strategy
217 or prophylaxis, and immunosuppressive protocols (triple immunosuppression including calcineurin-
218 inhibitors) play a role [19]. Protocols without mTOR inhibitors appear to delay CMV-specific
219 immune response and contribute to the onset of infection/disease in KT patients [20]; this has been
220 reported for lymphocyte-depleting agents (i.e. antithymocyte globulin) and calcineurin-inhibitor-

based regimens. In the present study, antithymocyte globulin was used only in six patients, therefore no conclusion can be drawn; whereas the association between non-responder status and the commonly used protocols including a calcineurin-inhibitor was only marginally significant. Previous studies have evidenced a reduced incidence of CMV events for everolimus-treated patients, in particular in the absence of prophylaxis [21-25]. Among the proposed mechanisms for anti-CMV activity of mTOR inhibitors, an action on antiviral CD8+ memory T-cell generation has been hypothesized [21].

An interesting finding was the higher frequency of non-responder status in men; it could be hypothesized that sex might have an effect on patterns of CMV-response *per se* or that this is due to differences in clinical features and management strategies (e.g., seronegativity more frequent in males, 13.8% versus 6.4%), as previously reported for some CMV indirect effects [26].

In conclusion, viro-immunological routine monitoring of CMV evidenced that restoration of specific T-cell response is frequently and stably achieved within few months posttransplantation and is associated to a favorable outcome in terms of reactivation risk. Levels of response of 20 SFU/2x10^5 CD3+ cells could be regarded as useful in terms of sensitivity and specificity for evaluating the risk of viral reactivation. A subgroup of KT can display a persistently non-responder status that could be due to other host-related determinants.

Based on this and considering the need for optimizing economic resources, the Turin Renal Transplant Centre has proposed the subsequent protocol for CMV immunological monitoring in KT: first evaluation at one month posttransplantation, in responders no further controls unless therapy modifications, rejection or CMV infection occur; in responders with the above mentioned conditions or non-responders, immunological evaluation in parallel to CMV-DNA up to 6 months or up to the development of a responder status with a re-control at 12 months. A single study using the QuantiFERON-CMV assay has also proposed the evaluation of pretransplant CMV responder status to stratify the risk of viral reactivation posttransplantation [27]. The usefulness of EliSPOT

assay in this context has not been yet investigated and specific investigations are needed. Further studies evaluating prophylaxis and pre-emptive treatment (in particular, the potential for defining cut-off levels for starting pre-emptive treatment also on the basis of immunological status) are needed, taking into consideration that a definitive evidence that immunological monitoring may guide successful clinical intervention or add value to virological monitoring is still lacking. Similarly, also the impact of immunosuppressive protocols, and other patient's and viral determinants in relation to CMV-specific immunological status should be further investigated in order to optimize the management of KT recipients.

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Figure 1. Rate of responders at different time points posttransplantation. For each time point, the median number of spot forming unit (SFU)/10^5 CD3+ cells is reported. Empty spaces in the graph are for time intervals. Number of patients tested as the individual time points: 169 at 30 days, 124 at 60, 108 at 90, 156 at 180, 148 at 360 (considering an overall number of 201 patients prospectively studied in the first year posttransplantation) and 127 patients at >360.

Figure 2. Occurrence of CMV-DNAemia in relation to CMV-specific cellular immune response. Percentages of patients with no episode, single episode or repeated episodes of CMV infection at follow up (mean 24.5 months, range 24-42) in responder and non-responder groups (Figure 2a). Mean viral load in the two groups; values are expressed as $\log_{10}$ copies/ml whole blood (peak level in each patient)(Figure 2b). Percentages of patients with CMV-DNAemia levels higher or lower than 10^5 copies/mL whole blood in the two groups (peak level in each patient)(Figure 2c).

Figure 3. Kaplan-Meier curve showing the time to occurrence of CMV viremia in responders (continuous line) versus non-responders (dotted line) who presented at least one episode of infection within 24 months posttransplantation.

Figure 4. Evaluation of operating characteristics for spot forming units (SFU/2 x 10^5 CD3+ cells) values in terms of occurrence of CMV-DNA in the subsequent 3 months by ROC curve analysis. 5 SFU/2x10^5 CD3+ cells: 51.4% sensitivity (95% confidence interval [CI] 41.5-61.3), 71.0% specificity (95% CI 66.6-75.1); 20 SFU/2x10^5 CD3+ cells, 71.4% sensitivity (95% CI 61.8-79.8), 60.0% specificity (95% CI 55.3-64.5); 100 SFU/2x10^5 CD3+ cells, 89.5% sensitivity (95% CI 82.0-94.5), 38.5% specificity (95% 34.1-43.1).

Table 1. Main features of study population.

	Total N= 328
Age, mean ± SD (range), years	54.7 ± 14.2 (28-75)
Gender	
Male	218 (66.5%)
Female	110 (33.5%)
CMV serological matching	
D+/R+	259 (79.0%)
D-/R+	30 (9.1%)
D+/R-	30 (9.1%)
D-/R-	9 (2.8%)
Time from transplantation	
< 1 year	201 (61.3%)
> 1 year (up to 8)	127 (38.7%)
Immunosuppression induction	
ATG	6 (1.8%)
Basiliximab	238 (72.6%)
Immunosuppressive protocol	
Including CNI	313 (95.4%)
Tac, MMF, steroid	177 (54.0%)
Tac, steroid	81 (24.7%)
CyA, MMF, steroid	17 (5.2%)
CyA, steroid	8 (2.4%)
Others	30 (9.1%)
Including mTOR inhibitors (everolimus, sirolimus)	28 (8.5%)
Antiviral strategy	
Prophylaxis (D+/R-)	30 (9.1%)
Pre-emptive therapy (D+/R+, D-/R+)	289 (88.1%)
None (D-/R-)	9 (2.8%)

N, number; SD, standard deviation; D, donor; R, recipient; ATG, antithymocyte globulin; CNI, calcineurin inhibitors; Tac, tacrolimus; MMF, mycophenolate mofetil; CyA, cyclosporine A; mTOR, mammalian target of rapamycin.

Table 2. Relation between demographic and clinical features of study population and CMV-specific cellular immune response status and viremia occurrence.

Feature	N	Non-responders N (%)	OR (95% CI)	p	CMV-viremia N (%)	OR (95% CI)	p
Sex							
M	218	82 (37.6)	0.5663	0.0286	71 (32.6)	0.9928	0.9769
F	110	28 (25.4)	(0.3404-0.9422)		36 (32.7)	(0.6090-1.6186)	
Age ≥ 50 years							
Yes	221	78 (35.3)	0.7822	0.3331	82 (37.1)	1.9350	0.0136
No	107	32 (29.9)	(0.4757-1.2863)		25 (23.4)	(1.1453-3.2690)	
Recipient CMV serostatus							
R+	289	82 (28.4)	6.4257	<0.0001	97 (33.6)	1.4651	0.3241
R-	39	28 (71.8)	(3.0570-13.5065)		10 (25.6)	(0.6858-3.1300)	
Anti-viral prophylaxis							
Yes (D+/R-)	30	22 (73.3)	0.1524	<0.0001	8 (26.7)	0.7309	0.4669
No (D+/R+, D-/R+, D-/R-)	298	88 (29.5)	(0.0654-0.3553)		99 (33.2)	(0.3142-1.7005)	
Time from transplantation							
< 1 year	201	69 (34.3)	0.9120	0.7024	75 (37.3)	0.8306	0.4657
> 1 year	127	41 (32.3)	(0.5687-1.4627)		32 (25.2)	(0.5044-1.3678)	
Immunosuppression induction with basiliximab							
Yes	235	90 (38.3)	0.4414	0.0042	101 (43.0)	10.9291	
No	93	20 (21.5)	(0.2521-0.7729)		6 (6.5)	(4.5940-26.0002)	< 0.0001
Immunosuppression induction with anti-thymocyte globuline							
Yes	6	1 (16.7)	2.5587	0.3938	2 (33.3)	1.0333	0.9701
No	322	109 (33.8)	(0.2952-22.1744)		105 (32.6)	(0.1863-5.7323)	
Immunosuppressive protocol including calcineurin-inhibitors (cyclosporine A, tacrolimus)							

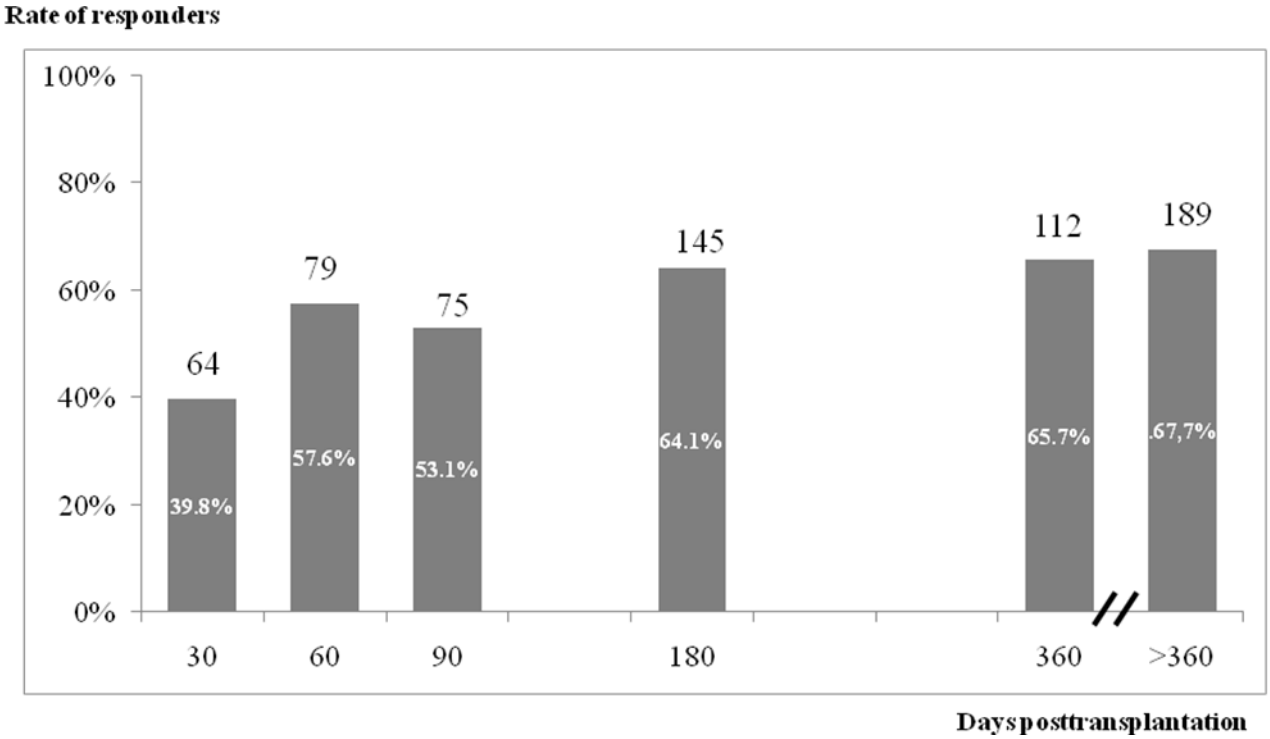
Yes No	313 15	110 (35.1) 0 (0)	0.0594 (0.0035-1.0024)	0.0502	105 (33.5) 2 (13.3)	3.2813 (0.7270-14.8104)	0.1223
Immunosuppressive protocol including mTOR-inhibitors (everolimus, sirolimus)							
Yes No	28 300	13 (46.4) 97 (32.2)	0.05513 (0.2525-1.2041)	0.1352	9 (32.1) 98 (32.7)	0.9764 (0.4262-2.2369)	0.9549
Total	328	110 (33.5)			107 (32.6)		

366

367

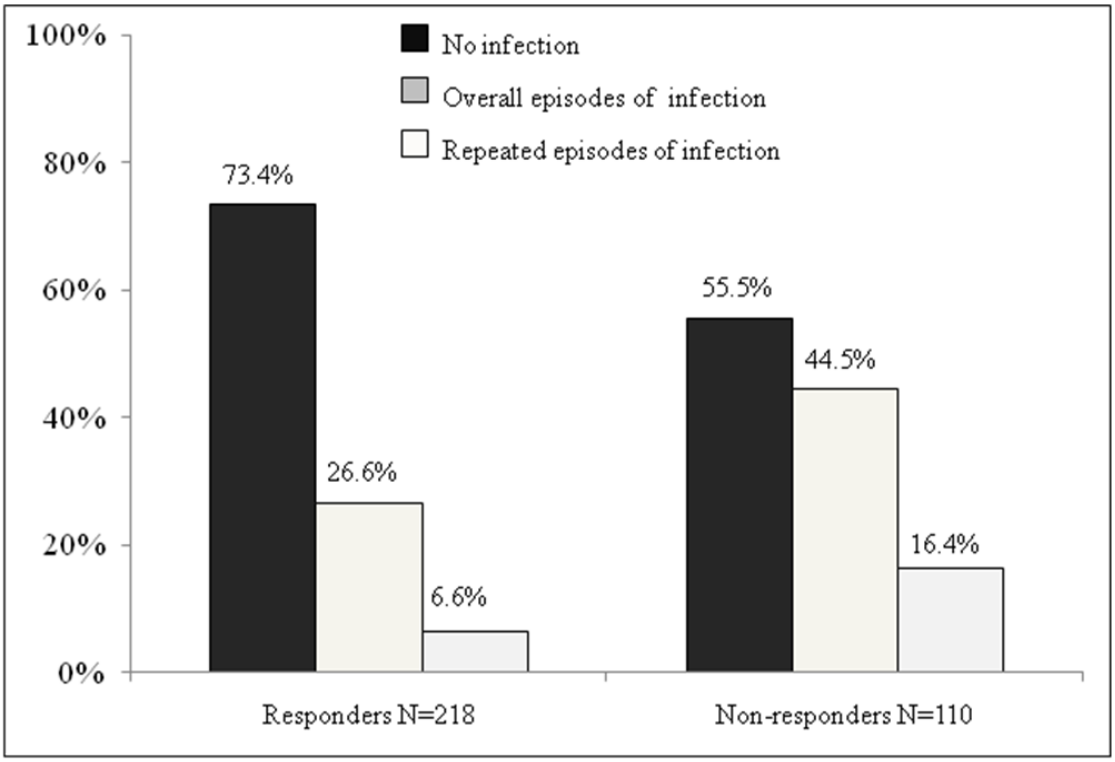
368 D, donor; R, recipient; OR, odds ratio; CI, confidence interval.

Figure 1


Rate of responders
100%
80%
60%
40%
20%
0%
64
79
75
145
112
189
39.8%
57.6%
53.1%
64.1%
65.7%
67,7%
30
60
90
180
360
>360
Days posttransplantation

375 **Figure 2**

376 **2a**



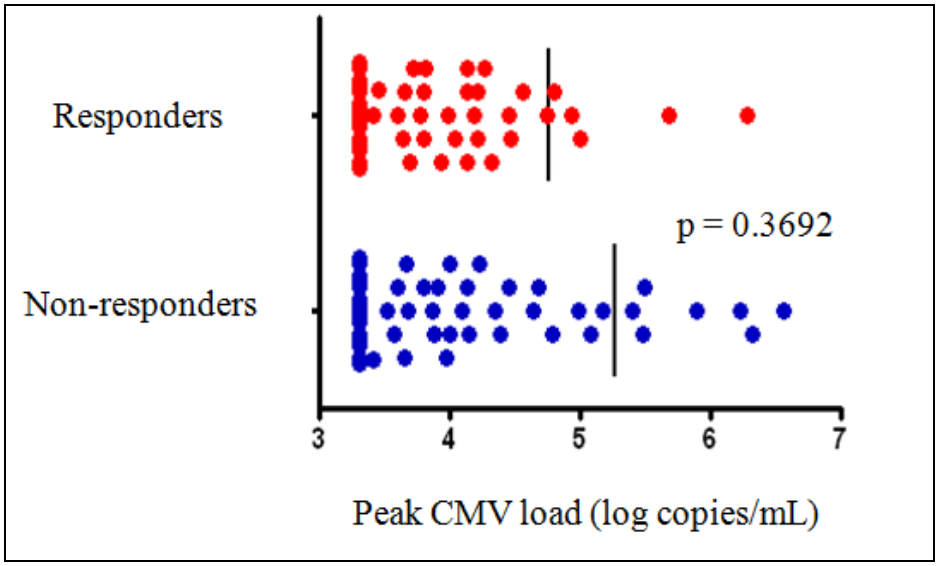
p = 0.002 for overall episodes of infection

p = 0.005 for repeated episodes of infection

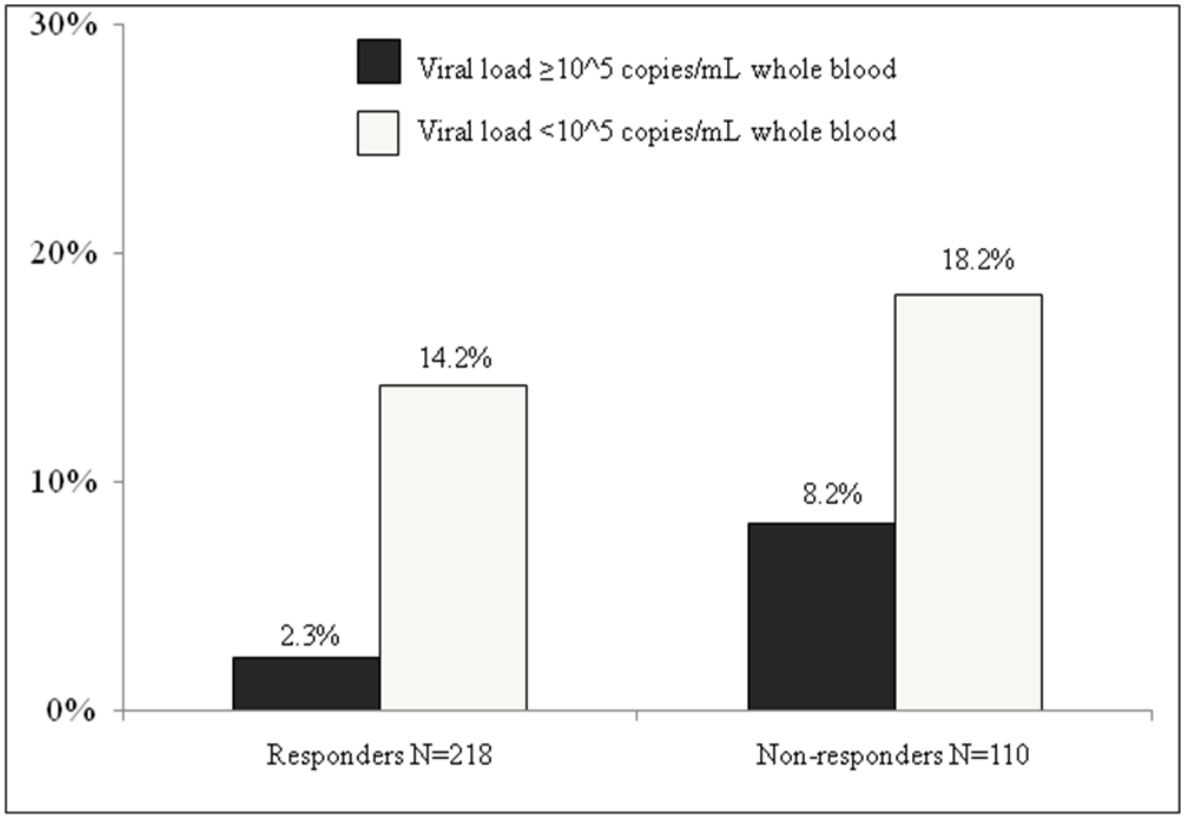
377

378

2b

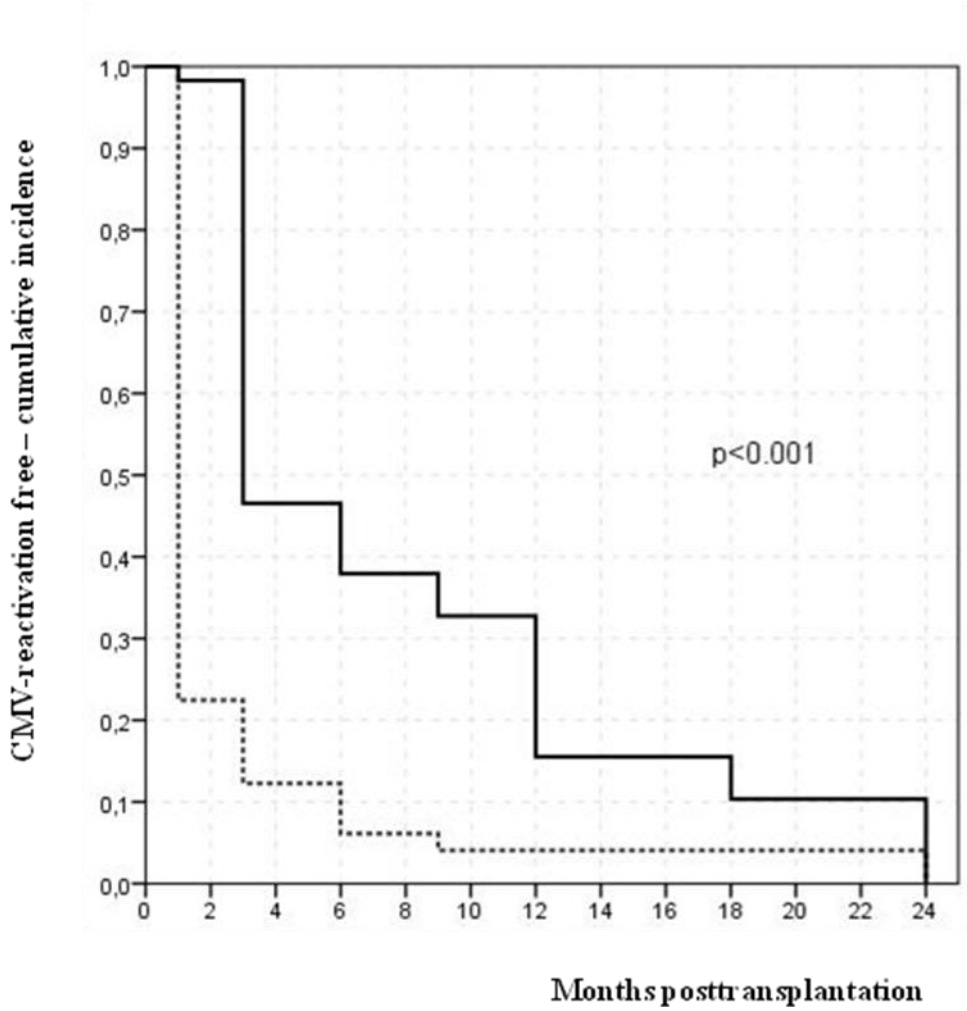

Responders
Non-responders
p = 0.3692
3
4
5
6
7
Peak CMV load (log copies/mL)

2c


30%
20%
10%
0%
Viral load ≥10^5 copies/mL whole blood
Viral load <10^5 copies/mL whole blood
2.3%
14.2%
8.2%
18.2%
Responders N=218
Non-responders N=110

p <0.05

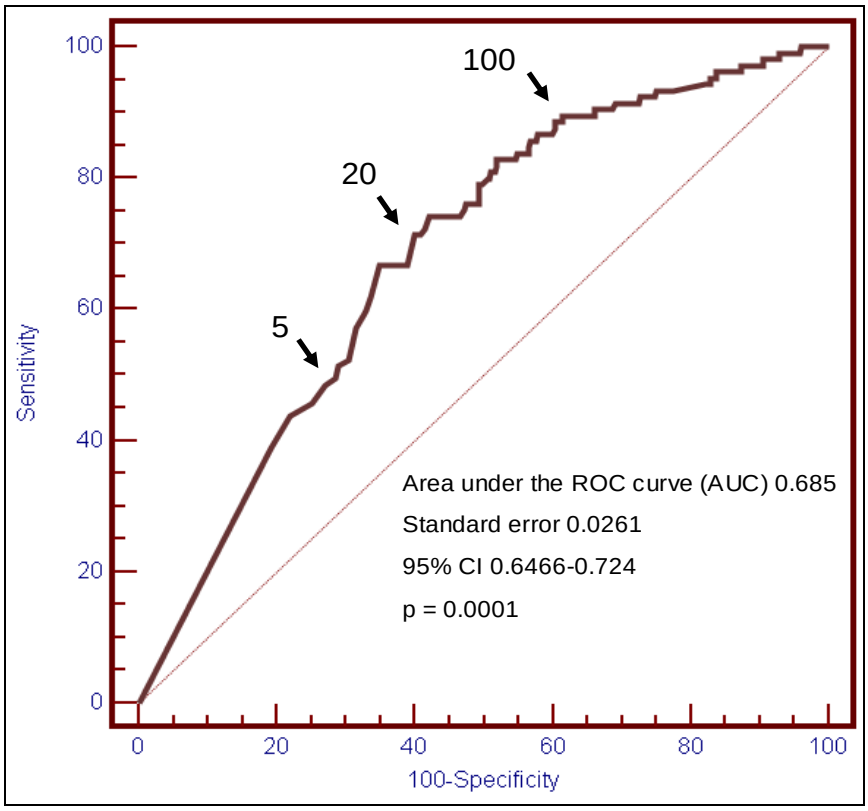
390 **3**


CMV-reactivation free – cumulative incidence
1,0
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
p<0.001
0
2
4
6
8
10
12
14
16
18
20
22
24
Months posttransplantation

391

392

4


100
80
60
40
20
0
Sensitivity
0
20
40
60
80
100
100-Specificity
100
20
5
Area under the ROC curve (AUC) 0.685
Standard error 0.0261
95% CI 0.6466-0.724
p = 0.0001